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GROWTH STUDIES WITH BACTERIA-FREE OYSTER (*CRASSOSTREA GIGAS*) LARVAE FED ON SEMI-DEFINED ARTIFICIAL DIETS[1]

C. J. LANGDON

College of Marine Studies, University of Delaware, Lewes, DE 19958

ABSTRACT

By the aseptic removal of gametes and *in vitro* fertilization of *Crassostrea gigas* eggs it was possible to obtain axenic larvae without the use of antibiotics. Optimal culture conditions for maintaining axenic larvae on algal diets have been developed.

Based on the results of ten growth experiments, *C. gigas* larvae fed on a semi-defined biphasic artificial diet had a mean shell length of 96.0 μm (standard deviation ± 3.0 μm) after 6 days of growth. This was significantly greater ($P < 0.001$) than the mean shell length of starved larvae (79.8 ± 0.8 μm), but was significantly less ($P < 0.001$) than that of larvae fed on the alga *Chaetoceros calcitrans* (113.6 ± 8.0 μm). *C. gigas* larvae grew on the dissolved organic fraction of the artificial diet alone, but growth was less than with the complete biphasic diet. This is the first reported demonstration that bivalve larvae can utilize dissolved nutrients for growth under axenic conditions where the possible nutritional contribution of bacteria is completely eliminated.

INTRODUCTION

One of the first steps in determining the nutritional requirements of an organism is the development of a completely defined artificial diet. However, no artificial diets have been reported that will sustain satisfactory growth of marine bivalves (*e.g.*, Winter, 1974; Masson, 1977). Castell and Trider (1974) reported that growth of *Crassostrea virginica* juveniles fed on semi-defined diets was only one-tenth that of animals kept in the sea.

One of the main difficulties in this area of research is the incompletely known but often deleterious effects of bacterial contamination associated with the culture of bivalves on artificial diets, since bacterial activity is increased by the addition of organic nutrients. Masson (1977), for example, observed that bacterial contamination may not only be directly harmful to bivalve larvae but may also cause food particles to form clumps that are too large for ingestion. On the other hand, bacteria may also serve as an undefined food source in nutrition studies (Zobell and Feltham, 1938; Martin and Mengus, 1977) thus making interpretation of the results virtually impossible.

There have been several successful attempts in obtaining axenic bivalve larvae with the use of antibiotics (Hidu and Tubiash, 1963; Millar and Scott, 1967). These techniques have not, however, been adopted by other workers, possibly because of the poor effectiveness of commonly used antibiotics, such as penicillin and streptomycin, against naturally occurring marine bacteria (Helm and Millican, 1977). In addition, other workers have shown that antibiotics may have adverse effects on

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the growth and survival of molluscs (Seneca and Bergendahl, 1955; Chernin and Schork, 1959).

This paper describes a method for obtaining axenic bivalve larvae without the use of antibiotics, together with the results of growth experiments with axenic *C. gigas* larvae cultured on artificial diets.

Materials and Methods

Obtaining axenic C. gigas *larvae*

Preliminary sterility tests using Droop's E6 sterility test medium (Droop, 1969) indicated that it was possible to obtain axenic gametes of *C. gigas* by aseptically removing them from the gonads of ripe adults. It was then possible to obtain viable, axenic *C. gigas* larvae by fertilizing the eggs *in vitro*.

The following protocol was developed in order to obtain axenic larvae for the nutrition experiments: Adult *C. gigas* were conditioned at 21°C for 1 month (Walne and Helm, 1974). After this period, the oysters were opened and a ripe male and female were selected. Under aseptic conditions, the surface of the gonad was wiped with 0.5% hypochlorite solution. A small incision was then made in the wall of the gonad and the gametes removed using a sterile Pasteur pipette. The eggs and sperm were collected separately in flasks containing 100 ml of autoclaved sea water at 25°C and 28 ppt salinity. The eggs, at a concentration of 50 eggs ml^{-1}, were fertilized by adding 100 to 300 sperm ml^{-1}. After fertilization, the eggs were incubated at 25°C for 18 to 24 h. The axenicity of the larvae was tested by adding a few milliliters of the larval suspension to one-tenth concentration of Droop's sterility test medium and incubating the medium at 25°C for 1 month. The development of a bacterial population in the sterility test medium indicated contamination of the larval culture. Eighty-five percent of more than one hundred batches of larvae, were shown to be axenic. The sterility of larvae obtained in this way was confirmed by epifluorescent microscopy using an acridine orange staining technique (Hobbie *et al.*, 1977). The axenicity of the larval cultures was also routinely tested at the end of each feeding experiment with Droop's sterility test medium and only the growth data from axenic cultures were used in the evaluation of the diets.

Axenic culture conditions

Larvae were cultured in the dark, in 100 ml flat bottomed flasks containing autoclaved natural sea water at 25 ppt salinity and 25–28°C. The cultures were agitated to maintain the artificial food particles in suspension. Bubbling was found to adversely effect the growth of larvae in the 100 ml culture flasks compared with the growth of larvae in standing flasks. Helm and Spencer (1972) have also reported a negative effect of bubbling on the growth of early straight-hinged *C. gigas* larvae. Best larval growth occurred in flasks which had a small conical protuberance in the center of the base and were agitated on an orbital shaker at 75 to 100 revs. min^{-1}. The protuberance prevented settled larvae and food from accumulating in the center of the base. Under these conditions, the mean shell length of larvae increased from 76.2 μm to 193.4 μm after 10 days of feeding on an algal diet of *Pyramimonas virginica*, compared with a final mean shell length of 118.0 μm for larvae grown in non-agitated flasks; the difference between the final mean shell lengths was statistically significant ($P < 0.001$).

For the growth experiments with artificial diets, the flasks were first baked at 450°C for 24 h to remove traces of organic material. The flasks were filled with 90

ml of sea water (25 ppt salinity), autoclaved at 115° for 15 min and then agitated on an orbital shaker for 48 h to restore the pH to 8–8.2 and oxygen and carbon dioxide concentrations to normal sea water levels. Larvae were added to the flasks to give a concentration of 2 larvae ml^{-1} and then the artificial diets were added. The cultures were then agitated in the dark on an orbital shaker at 25–28°C. Food particles (2.2–12.6 μm in diameter) were added to the flasks periodically to maintain a particle concentration of between 20 and 100 particles μl^{-1}. The growth experiments were carried out over a period of 6 days. The culture medium was not changed during the experimental period.

At the end of the experiment, the larvae were sieved from the culture medium using a 35 μm mesh screen and preserved in 1% formalin solution (made up in 25 ppt salinity sea water, pH 8.0). The shell lengths of at least 100 larvae from each culture were measured. The mean shell length and standard deviation were calculated and comparisons among treatment means were made using Student's *t*-test.

Preparation and development of an artificial diet

A biphasic diet was developed for *C. gigas* larvae which consisted of a soluble phase and a particulate phase, the latter made up of co-precipitated egg albumin and starch particles. Biphasic diets have been used successfully in the axenic culture of the brine shrimp *Artemia salina* (Provasoli and d'Agostino, 1969) and the water flea *Moina macrocopa* (Conklin and Provasoli, 1977, 1978). The gross composition of the particle developed for *C. gigas* larvae was based on the composition of the alga *Pavlova lutheri*, as reported by Parsons *et al.* (1961), which is a satisfactory algal food species for *C. gigas* (Millican and Helm, 1973). The composition of the particle is given in Table 1.

The particle was prepared by firstly heating and dissolving 150 mg of rice starch in 20 ml of distilled water. The solution was placed on a magnetic stirrer, a stream of nitrogen was introduced, and 50 mg of an oyster lipid extract, dissolved in 2 ml of chloroform, were added. The lipid extract was prepared by homogenizing the body tissues of adult *C. gigas* in chloroform/methanol according to the method of Bligh and Dyer (1959). The chloroform was removed with gentle heating. Next 59.3 mg of $Na_2HPO_4 \cdot 2H_2O$ (equivalent to 10 mg of phosphorus), 200 mg of egg albumin (Sigma, Fraction V) dissolved in 5 ml of 1 *M* NaCl, together with 1 mg each of RNA (Sigma, from yeast) and DNA (Sigma, from salmon sperm) dissolved in a minimum volume of 1 *M* NaOH were added. The mixture was poured into 70 ml of boiling sea water in order to precipitate the particle, and the pH of the suspension adjusted to pH 8.0 with 1 *M* NaOH. The precipitate was then autoclaved and a fine particulate suspension was prepared by homogenization with a sterile glass tissue grinder under aseptic conditions.

The composition of the water-soluble phase of the diet was initially based on that of tissue culture medium (TCM) 199 and was prepared according to Morgan *et al.* (1955). All the soluble nutrients of TCM 199 were sterilized by 0.2 μm Millipore filtration. It was found that the concentrations of the dissolved nutrients of TCM 199, recommended for the culture of animal tissues, were too high for oyster larvae and caused both swelling and protrusion of the velum from between the shell valves. This condition resulted in tissue loss and death of the larvae. At one-hundredth the recommended concentration of TCM 199, larvae grew without the development of such morphological abnormalities.

In subsequent experiments, several alternatives to the TCM 199 amino acid mixture were tested at different concentrations. These mixtures were based on the

TABLE I

Composition of the final artificial diet developed for axenic Crassostrea gigas *larvae*

Co-precipitated particle. Fed at a concentration of 100 particles μl^{-1}

	Composition, ratio by wt.
Egg albumin (Fraction V, Sigma)	200
Rice starch	150
Oyster lipid extract	50
Phosphorus (as $PO_4^{///}$)	10
R.N.A. (yeast)	1
D.N.A. (salmon sperm)	1

Dissolved nutrients

[a] *Amino acids*	*mg* l^{-1}
Alanine	0.97
DL-2-amino-iso-butyric acid	0.05
DL-2-amino-n-butyric acid	0.13
Arginine (Cl)	0.57
Aspartic acid	0.99
Cysteine (Cl)	0.05
Glutamic acid (H_2O)	0.84
Glycine	0.63
Histidine (Cl)	0.19
iso-Leucine	0.33
Leucine	1.02
Lysine (Cl)	0.73
Methionine	0.32
DL-Ornithine	0.04
Phenylalanine	0.44
Proline	0.67
Serine	0.60
Threonine	0.50
Tryptophan	0.04
Tyrosine (Na_2 salt)	0.21
Valine	0.68

Glucose, water and fat-soluble vitamins, purines, pyrimidines and other water soluble components were added at 1/100 the concentration recommended for tissue culture medium 199 (Morgan *et al.*, 1955).
1% v/v bovine amniotic fluid (Gibco) was added as a beneficial, undefined source of nutrients.

[a] Amino acid mixture based on the composition of the alga *Isochrysis galbana*, according to Chau *et al.* (1967). All amino acids were L-isomers unless otherwise indicated.

composition of the culture medium for *A. salina* (AA 1A mix; Provasoli and d'Agostino, 1969) and for *M. macrocopa* (Conklin and Provasoli, 1977, 1978) as well as a mixture based on the composition of the alga *Isochrysis galbana* (after Chau *et al.*, 1967). Of these, the *Isochrysis* mixture (see Table I), at a total amino acid concentration of 10 mg l^{-1}, resulted in significantly greater larval growth ($P < 0.001$) than with the other mixtures tested. It was therefore used instead of the TCM 199 amino acid mixture in the artificial diet. The amino acid mixture developed for *A. salina* was harmful to the oyster larvae at the concentration used by Provasoli and d'Agostino (1969), and caused swelling of the velum and tissue loss.

A range of water soluble vitamin mixtures was also tested to find substitutes for the TCM 199 vitamin mixture at different concentrations. These included Conklin

and Provasoli's (1977, 1978) mixture for *M. macrocopa* and the ASP_2 vitamin mixture reported by Provasoli *et al.* (1957) for the culture of marine algae. Neither of these was superior to the TCM 199 vitamin mixture at one-tenth the recommended concentration, and the vitamin mixture described for *M. macrocopa* was harmful to oyster larvae both at one-tenth and at the full concentration reported to support good growth of this crustacean species. Later experiments indicated that the best larval growth occurred if the concentration of the water soluble vitamins of the TCM 199 medium was reduced from one-tenth to one-hundredth that of the recommended concentration for the culture of mammalian cells (Morgan *et al.*, 1955).

Apart from the defined nutrients of the culture medium developed for oyster larvae, it was found that the addition of 1% v/v bovine amniotic fluid (Gibco) to the artificial diet significantly improved larval growth ($P < 0.05$). This undefined nutrient source was commercially available as a sterile solution. The composition of the final oyster culture medium is given in Table 1.

Results

Growth of larvae fed on the final artificial diet of Table I

Based on the results of ten separate experiments, larvae fed on the final artificial diet (Table I) had a mean shell length of 96.0 μm (standard deviation $\pm$ 3.0 μm) after 6 days of growth. This was significantly greater ($P < 0.001$) than the mean shell length of starved larvae (79.8 $\pm$ 0.8 μm), but was significantly less ($P < 0.001$) than that of larvae fed on the alga *Chaetoceros calcitrans* (113.6 $\pm$ 8.0 μm). Most of the larvea fed on the artificial diet reached the umbone stage of development within 6 days, but little further growth occurred, and after 8 days tissue loss became increasingly apparent. Feeding experiments indicated that the larvae fed on the artificial diet cleared very little of the suspended particulate fraction, and the guts of approximately 80% of the larvae were empty.

Larvae fed on mixtures of algae and artificial nutrients

It was difficult to determine whether the poor feeding activity of the larvae on the artificial food particles was due to deficiencies in the nutritional quality of the diet or due to some growth inhibitory effect of the dietary components. An experiment was therefore carried out in which the particulate and dissolved fractions of the diet were tested singly or in combination with an algal diet. Any inhibitory effect of either of these two fractions on the growth of the larvae would, therefore, become evident. For this experiment, *Dunaliella tertiolecta* was chosen as the algal food since it was available in axenic culture and was reported to be of only moderate food value for oyster larvae (Walne, 1963); therefore, there was possible scope for improvement of the algal food by the addition of supplements of artificial nutrients.

The results (Table II) indicated that in the absence of *D. tertiolecta* the larvae grew best on the complete artificial diet. Larvae grown on the dissolved organic fraction alone were significantly larger ($P < 0.001$) than starved larvae after 6 days of culture. The particulate fraction of the diet did not support larval growth and tissue loss and high larval mortality was evident. In combination with *Dunaliella*, the complete artificial diet had little effect on larval growth compared with the growth of larvae fed on *Dunaliella* alone. However, addition of the dissolved organic fraction of the artificial diet to *Dunaliella*, significantly improved larval growth ($P < 0.001$) compared with the growth of larvae fed on algae alone. Addition of the

TABLE II

Growth of axenic C. gigas *larvae fed on components of the artificial diet (Table I) alone or in combination with the alga* Dunaliella tertiolecta *(25 cells μl⁻¹)*

Artificial diet	[a] Mean shell length ± s.d. (μm) Without algae	With algae
None	80.9 ± 0.8	97.1 ± 1.2
Particulate fraction	Larvae died, vela protruding	Larvae died, vela protruding
[b] Dissolved fraction	95.3 ± 1.0	123.6 ± 2.3
Dissolved plus particulate fraction	98.9 ± 0.8	97.7 ± 1.2

Initial larval shell length = 74.7 ± 0.5 μm.

[a] Based on two replicate cultures for each treatment and 100 larvae measured from each culture.
[b] Dissolved organic fraction consisted of the defined components given in Table I plus 1% v/v bovine amniotic fluid.

particulate fraction of the diet to *Dunaliella* caused protrusion of the vela and high larval mortality.

Testing alternative food particle types to the co-precipitated egg albumin and starch particles

The results of the growth and feeding experiments indicated that the co-precipitated egg albumin and starch particle was not a satisfactory means of feeding the larvae on protein and carbohydrate. Therefore a range of alternative food particle types were tested (see Table III). Red blood cells were selected for testing because Claus and Adler (1970) reported good growth of both *C. virginica* larvae and spat fed on this food. Since Conklin and Provasoli (1977, 1978) reported the successful

TABLE III

Growth of C. gigas *larvae cultured on the dissolved organic fraction of the artificial diet (Table I) and particulate foods[a] under axenic conditions*

Diet	Mean shell length + s.d. (μm)
Controls	
Starved larvae	79.9 ± 0.8
Algal-fed larvae[b] (non-axenic)	99.6 ± 1.8
Dissolved organic fraction (DOF) alone	87.7 ± 1.1
DOF + egg albumen/starch particles	94.3 ± 1.2
Treatments	
DOF + defibrinated blood cells (calf)	86.7 ± 1.1
DOF + bovine serum albumin/amylose particles	90.6 ± 0.9
DOF + heat precipitated bovine amniotic fluid[c]	90.7 ± 0.9
DOF + raw whole egg particles	91.9 ± 1.1[d]
DOF + raw egg yolk particles	94.0 ± 1.1[d]
Initial larval shell length	75.4 ± 0.4 μm

[a] All particles were added at concentrations of 100 particles μl^{-1}.
[b] Algal food was *Chaetoceros calcitrans* at an initial concentration of 100 cells μl^{-1}.
[c] Added at a concentration of 1% v/v.
[d] >75% larvae were in poor condition with protruding vela and loss of body tissues.

use of particles prepared with egg yolk or bovine serum albumin in the culture of *M. macrocopa*, particles prepared with these nutrients were also tested. The bovine serum albumin/amylose particles were prepared by co-precipitation with heat in a similar way to the egg albumin and starch particles (see Methods section).

None of the food particle types supported larval growth better than the co-precipitated egg albumin and starch particles ($P < 0.05$) (Table III). Furthermore, both whole egg and egg yolk had an adverse effect on the larvae and caused loss of the body tissues and protrusion of the velum. Larval growth on the dissolved organic fraction of the diet alone was statistically greater ($P < 0.001$) than that of starved controls, and was further improved by adding the egg albumin and starch particles to the dissolved organic fraction; these results therefore confirmed the findings of the previous experiment (Table II).

Discussion

A method for reliably obtaining large numbers of axenic *C. gigas* larvae without the use of antibiotics has been described. The method need not be restricted to oysters since it can be applied to animals from which axenic gametes can be removed and fertilized *in vitro* under aseptic conditions. The ability to eliminate bacteria from nutrition studies of marine filter-feeders is an essential step in the development of defined artificial diets for these animals. This method may also be useful in studies of the interaction between marine invertebrates and bacteria.

The feeding experiments indicated that the culture media developed for *A. salina* (Provasoli and d'Agostino, 1969) and *M. macrocopa* (Conklin and Provasoli, 1977, 1978) was not suitable for *C. gigas* larvae since the larvae would not tolerate high concentrations of the dissolved nutrients. Even so, the lower concentrations of the dissolved nutrients of the artificial diet developed for *C. gigas* were much higher than those occurring in natural conditions. For example, the concentration of free amino acids in the dissolved organic fraction of the diet (10 mg l^{-1}) was about one thousand times greater than those reported to occur naturally in oceanic waters (Williams, 1975). Furthermore, co-precipitated egg albumin and starch particles, which were similar to those developed for *A. salina* and *M. macrocopa*, caused protrusion of the velum, tissue loss, and high mortality when fed to the larvae in the absence of the dissolved organic fraction of the diet (Table II). However, in combination with the dissolved organic fraction the particles had a positive effect on growth (Table II and III), although very little ingestion of the particles was apparent.

It is not clear why the adverse effect on the larvae of the co-precipitated egg albumin and starch particles depended on the absence of the dissolved organic fraction of the diet (Table II). Adverse effects were also evident with raw whole egg and raw egg yolk (Table III) as well as with other nutrients tested but not reported here such as calf serum, lobster haemolymph, casein, and freeze dried or heat killed algal cells (see Langdon, 1980). All these nutrients were similar in that they were largely or wholly made up of protein. It is probable that particulate or dissolved foreign proteins had a toxic effect on larvae which was apparent in the protrusion of the velum and high mortality. Further testing of this hypothesis is necessary.

The dissolved organic fraction of the artificial diet (Table I) supported larval growth that was significantly greater ($P < 0.001$) than that of starved controls (Table II, III). This is the first demonstration that marine bivalve larvae can utilize dissolved nutrients for growth under axenic conditions where the possible nutritional contribution of bacteria is completely eliminated.

There is now conclusive evidence that there is a net uptake of dissolved amino acids from sea water at naturally occurring concentrations by adult mussels *Mytilus edulis* (Manahan *et al.*, 1982). Manahan and Crisp (1982) have also demonstrated that *C. gigas* larvae take up dissolved ^{14}C-labelled amino acids from sea water and either respire the ^{14}C as $^{14}CO_2$ or incorporate the ^{14}C into macromolecules in the body tissues. Uptake is via the velum and not via the gut (Manahan and Crisp, 1982) and therefore, in oyster larvae, uptake of nutrients via the velum compliments uptake of the nutrients via the gut.

The beneficial growth effect of the dissolved organic fraction of the artificial diet was also evident when it was added as a supplement to the alga *Dunaliella*, compared with the growth of larvae fed on *Dunaliella* alone (Table II). Whether larval growth was enhanced directly by uptake of the dissolved organic fraction or indirectly via prior algal uptake, is not known. Davis and Chanley (1955) also reported improvement in the growth of *C. virginica* and *Ostrea edulis* larvae fed on an algal diet supplemented with a vitamin mixture. D'Agostino and Provasoli (1968) found an improvement in the growth and development of *A. salina* when vitamin supplements were added to algal diets and Murphy (1970) noted similar findings with various species of *Daphnia*.

In conclusion, *C. gigas* larvae required both particulate and dissolved organic nutrients for maximum growth when fed on the artificial diet tested. Further research is required, however, in order to improve the methods of delivering dietary components to the larvae. Co-precipitated egg and starch particles are not satisfactory since they are poorly ingested and have an inhibitory effect on growth when fed to the larvae in the absence of the dissolved organic fraction of the diet. Recent techniques for delivering dietary protein in alginate or carboxymethyl cellulose gel particles (Langdon, 1983) are proving to be advantageous. When a satisfactory method for delivering nutrients to bivalve larvae is found then the effects of the nutritional composition of artificial diets on oyster growth can be tested with greater rigor. With axenic culture conditions, it will be possible to chemically specify the nutritional requirements of bivalve larvae and to better understand their nutrition in both natural and artificial conditions.

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